BACTEROIDES FRAGILIS IN SEPSIS – MICROBIOLOGICAL STUDIES

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CERTIFICATE

This is to certify that the dissertation entitled **"BACTEROIDES FRAGILIS IN SEPSIS – MICROBIOLOGICAL STUDIES"** by **Dr. C. SUGUMARI**, for M.D. Microbiology Examination, March 2007 under The Tamil Nadu Dr. M.G.R. University is a bonafide work carried out under my direct supervision and guidance.

Director

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DECLARATION

I, **Dr. C. SUGUMARI** declare that the dissertation titled **"BACTEROIDES FRAGILIS IN SEPSIS – MICROBIOLOGICAL STUDIES"** has been prepared by me.

This is submitted to The Tamil Nadu Dr. M.G.R. Medical University, Chennai, in partial fulfilment of the requirement for the award of M.D. Degree, Branch IV (MICRO BIOLOGY) degree Examination to be held in MARCH 2007.

Place : Madurai

Date : **Dr. C. SUGUMARI**

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CONTENTS

INTRODUCTION

Bacteria, the oldest forms of life on earth, are remarkably diverse and exist in astounding number. Diseases caused by bacteria include some of the most common infections in the world, as well as some of human kinds most important scourges, past, present and probably future. At the same time, each of us is colonized by more bacterial cells than we have human cells in our bodies. Generally, this is a peaceful even productive (symbiotic) relationship, but occasionally even these well tolerated residents of the human biosphere cause disease.[53]

Infections may be caused by Gram positive or Gram negative bacilli or cocci [81], and these were the first recognized bacterial agents of disease. In recent years, classification has been dominated by genotype especially relying on conserved molecules like 16SrRNA. In the future, taxonomy, understanding of pathogenesis and diagnostics will be increasingly based on genotype. Thus the knowledge of taxonomy and molecular biology must be broadened.

BACTERIAL EVOLUTION

The Study of infectious diseases is a dynamic field at least in part because of the changing nature of the pathogens we consider. An obvious and absolutely critical aspect of bacterial evolution is the acquisition of resistance to antimicrobial agents [67]. Understanding resistance patterns is pivotal to understand proper therapeutic approaches. Also importantly understanding the biology, epidemiology and mechanisms for resistance leads to the ways we will need to prevent and curtail resistance in the population of microbes that infect us, as well as those that colonize us which may provide reservoirs of resistance. Resistance [15] is among the easiest phenotypes to detect and understand

Based on the influence of oxygen and viability, bacteria are divided into aerobes and anaerobes [53]. Aerobic bacteria require oxygen for growth and anaerobic bacteria grow in the absence of oxygen which may be further sub divided into facultative anaerobes which are ordinarily aerobic but can also grow in the absence of oxygen though less abundantly obligate anaerobes may even die on exposure to oxygen

Anaerobic bacteria are major components of human microflora residing on mucous membrane [29] and predominate in many infectious processes particularly those arising from mucosal sites. Thus organisms generally cause disease subsequent to the break down of mucosal barriers

and the leakage of indigenous polymicrobial flora into normally sterile sites.

The predominance of anaerobes in certain clinical syndromes can be attributed to the large number of these organisms residing on mucus membranes [29], the elaboration of a variety of virulence factors, the ability of some anaerobic species to resist oxygenated microenvironments [8], synergy with other bacteria and resistance to certain antibiotics [13].

DEFINITION OF ANAEROBE

An anaerobe is an organism that requires reduced oxygen for growth. Practically, this means that an anaerobe fails to grow on the surface of solid media in 10% of carbondioxide in air. As opposed to the strict requirement for survival only under anaerobic condition of several anaerobic species inhabiting our bodily surface, anaerobes that commonly cause human infections are aerotolerant [8] and can survive for as long as 12 hours in the presence of an oxygenated atmosphere, although they will not grow. Most anaerobes do not possess catalase but those that cause human disease often have superoxide dismutase [15] .

ROLE OF ANAEROBES AS NORMAL FLORA

Several hundred species of anaerobic organisms have been identified in the human microflora [8]. Mucosal surfaces such as the oral cavity, Gastro Intestinal Tract, the female genital tract are the major reservoirs for this group of organisms [29]. It is interesting to note that

anaerobes inhabit areas of the body that are exposed to air via, the skin, nose, mouth and throat. It has been hypothesized that anaerobes can withstand oxygen at these sites, in part owing to the presence of aerobes and facultative organisms, which consume oxygen and reduce oxidation reduction potential [13, 52]. They are also believed to reside in areas of those sites that are more protected from oxygen such as gingival crevices [29].

Despite the number of anaerobic species found in normal flora, relatively few are involved in human infections. Infections involving anaerobes are polymicrobial [8] in nature and usually result from disruption of mucosal surfaces and the subsequent infiltration of resident flora. However certain Gram negative anaerobic bacilli belonging to the genera of Bacteroides, Fusobacterium, Porphyromonas and Prevotella are most commonly isolated from clinical infections [65].

The occupation of distinct ecological niches within the intestinal environment that would otherwise be filled with potentially pathogenic organisms is among the most important roles that anaerobes serve as normal colonic microflora [15]. This process **"COLONISATION RESISTANCE"** effectively interferes with colonization by potentially pathogenic bacterial species through the depletion of oxygen and nutrients and production of enzymes and the toxic end products [8]. The anaerobic component of the intestinal microflora is also responsible for

the production of secreted products, like Vitamin K and bile that are helpful in human health.

The hallmark of infection caused by these Gram negative anaerobic bacteria is abscess formation [8,11,21,61] . The predominant Gram negative anaerobes include Bacteroides fragilis [65], Prevotella species, Fusobacterium species, Porphyromonas species. Although some of these organisms are numerically dominant in the normal flora [29], others make up a much smaller proportions indicating that they possess one or more virulence factors that contribute to their ability to cause disease.

The Bacteroides fragilis group consists of Gram negative bacilli that are among the anaerobes most commonly isolated from human infections [10]. This group comprises several species including Bacteriodes fragilis, Bacteriodes thetaiotaomicron, Bacteriodes ovatus, Bacteriodes vulgatus, Bacteriodes uniformis, Bacteriodes diastonis. From this group, Bacteroides fragilis is the species most commonly isolated [15, 65] from clinical cases particularly in infections emanating from the lower intestine although other members of this family are also isolated from infectious sites [40].

BACTEROIDES FRAGILIS

The anaerobic Gram negative bacilli that make up the genus Bacteroides are among the most important constituents of the normal human flora and are plentiful in the oral cavity, GIT, and the vagina [29]. At

one time the genus Bacteroides consisted of almost 50 species , but many of the species have now been transferred to new genera. The genus Bacteriodes now consists of species previously categorized into the B.fragilis group and some closely related species. B.fragilis, the most important member of this genus, is pleomorphic in size and shape resembling a mixed population of organisms in a casually examined Gram stain. Bacetroides have a typical gram negative cell wall structure, which can be surrounded by a polysaccharide capsule [15,47,86]. A major component of the cell wall is a surface lipopolysaccharide.

Among the common infections caused by these bacteria are periodontal disease, post aspiration pleuropulmonary infection [20] , genital tract infection in women [73] and intra abdominal abscesses [23] , CNS infections like brain abscesses and rarely meningitis [17, 41], bacteremia [31, 50], bone and joint infections [9, 38] and skin & soft tissue infections such as diabetic [9, 13, 34, 52] and decubitus ulcers [9]. Cutaneous absess below the waist have often been found to be caused by colonic flora anaerobes including Bacteroides fragilis.

These bacteria are identified presumptively on the basis of colony morphology, Gram staining characteristics, pigment production, susceptibility to special strength antibiotic disc, and biochemical tests. Definitive identification requires multiple biochemical tests which are tedious to perform and because of expense, not feasible for most clinical

laboratories. Because of its clinical importance and relative antimicrobial resistance, identification of B. fragilis is essential. B fragilis group can be distinguished from other species of anaerobic Gram negative bacilli by growth in 20% bile [89] and resistance to special strength antibiotic disc [22] like kanamycin, vancomycin and colistin.

MICROSCOPIC APPEARANCE

They are long, slender, nonsporing, encapsulated, pale irregular staining, pleomorphic (coccobacillary), saccharolytic, Gram negative bacilli [22]. They may be Gram variable with irregular or bipolar staining.

CULTURAL CHARACTERS

A combination of non selective, selective media [76] is used for the isolation and presumptive identification of this bacteria from clinical material. A selective medium is required to isolate Gram negative anaerobes from specimens which may contain contaminating facultative flora. They grow on 5%sheep blood agar and bile esculin agar with kanamycin. On 5% sheep blood agar, the colonies are grey white, glistening, non haemolytic, 1-3 mm diameter usually within 48 hours of anaerobic incubation at 37^0C. Colonies of Bacteroides fragilis on bile esculin agar with kanamycin will be grey, circular, entire, raised, more than 1 mm diameter with stippling in the medium around the colonies.Most aerobic and anaerobic bacteria are inhibited by bile and

kanamycin in this medium, whereas Bacteroides fragilis is stimulated by bile , resistant to kanamycin and able to hydrolyse esculin [82].

BIOCHEMICAL IDENTIFICATION

B. fragilis are catalase positive [87, 89] and spot indole negative. They can ferment sugars among which sucrose and arabinose are used to differentiate B.fragilis from other bile resistant species of B.fragilis group B.fragilis is sucrose positive and arabinose negative [82].

VIRULENCE FACTORS ASSOCIATED WITH B.FRAGILIS

1. capsular polysaccharide [15] - B.fragilis has an immunologically distinct capsule[47] composed of two polysaccharide Polysaccharide–A, Polysaccharide – B [86].

2. The adherence of B.fragilis to intestinal epithelium and mucous is promoted by pili [15].

3. Lipopolysaccharide endotoxin [15] formed by B.fragilis contributes to abscess formation , lacks lipid A and is biologically impotent.

4. B.fragilis produces short chain fatty acid, succinic acid [9,15], which inhibits phagocytosis through this product.

5. Enzymes - variety of enzymes produced by Bacteroides species may contribute to tissue damage or enable these pathogens to escape host defenses. Among these are hyaluronidase, collagenase, neuraminidase, heparinase, fibrinolysin [9, 15].

Many clinically important anaerobes including B.fragilis have some degree of oxygen tolerance, presumably through production of the important enzyme superoxide dismutase. Cell mediated immunity surprisingly appears to be more important with regard to B.fragilis [43]. By lowering the redox potential in the microenvironment, facultative organisms may promote more favourable condition for anaerobic growth and that the anaerobic component in mixed infection may facilitate the growth of facultative bacteria through the inhibition of phagocytosis (SYNERGY) [69].

ANTIMICROBIALS

The antimicrobials used to treat anaerobic infections should have activity against both aerobic and anaerobic organisms [52] as many of these infections are of mixed etiology [15]. Antimicrobials with greatest activity against nearly all anaerobic bacteria include carbapenems, betalactamase inhibitor combinations, metronidazole and chloramphenicol .

RESISTANCE

The medically important Bacteroides species are typically resistant to penicillin. Treatment failure with penicillin or first generation cephalosporins is common for infections that involve B.fragilis [15] .

Metronidazole, a 5-nitro imidazole derivative, is the drug of choice for treatment of Bacteroides infection [45,74], as resistance is rarely

reported. It acts by reduction of its 5-nitrogroup [15] which is cytotoxic and causes strand breakage of DNA, thereby causing death of the cell. It has radiosenstising effect on the hypoxic tumour cells. The penetrating property of metronidazole into the abscesses and the necrotic tissues, where anaerobic organism thrive has revolutionized the therapy in such conditions. Along with other antimicrobial agents, it is highly effective in post operative deep seated anaerobic infections which are polymicrobial in nature [52]. The failure of antibiotic therapy against an anaerobic infection should prompt consideration of surgical intervention. In addition, the possibility of co infection with a drug resistant organism is to be considered.

Antimicrobial resistance in B.fragilis was first recognized in the late 1960's and early 1970's. Until the mid 1980 , almost all clinical isolates of Bacteroides appeared to be susceptible to metronidazole , with the exception of rare strains such as those described by Ingham et al, in 1978. Fortunately enough, incidence of resistance to metronidazole remains low (<5%) [66]. A major contributing factor in the emergence of metronidazole resistant Bacteroides species is the acquisition and transfer of antibiotic resistance via chromosomal or on mobilisable plasmids [15, 24]. The mechanisms of metronidazole resistance in Bacteroides is due to reduced uptake of drug, reduced nitroreductase activity, decreased pyruvate, ferredoxin oxidoreductase activity and increased lactate

dehydrogenase activity. The transfer of metronidazole resistance is due to 7.7Kb plasmid, and in few strains chromosomal [15].

PCR AND DNA SEQUENCING

The DNA sequencing of the purified PCR product [24,91] is shown to be an useful method to find out resistance genes [45], which are found to be 'nim'resistance genes [33, 37]. The first metronidazole resistant Bacteroides isolates were found [46, 66, 80, 85] to have altered end products of glucose metabolism. The characterization of the 'nim' gene [79], revealed a more prevalent mechanism for metronidazole resistance, either chromosomal or on mobilisable plasmids [15], encoding a nitroreductase that catalyses drug uptake and reduction without the formation of damage inducing nitro radicals.

AIM AND OBJECTIVES OF THE STUDY

- To find out the prevalence of Bacteroides fragilis among patients with sepsis.
- To perform antimicrobial susceptibility testing of Bacteroides fragilis.
- To identify metronidazole resistant Bacteroides fragilis.
- To confirm the resistant pattern of Bacteroides fragilis genomically by short gene sequencing.
- To correlate with clinical conditions.
- To provide guidelines to clinicians in treating cases with sepsis.

REVIEW OF LITERATURE

Mandell [53], "PRINCIPLES AND PRACTICE OF INFECTIOUS DISEASE " V EDITION, VOLUME 2; CHAPTER 233; ANAEROBIC BACTERIA – GENERAL CONCEPTS.

The first evidence that microorganisms might live under anaerobic conditions was that of Antonie van Leeuwenhoek, who noted that some of his 'animalcules' were able to live and move about in the absence of air. The phenomenon of anaerobiosis was more clearly described by Louis Pasteur in 1861, when he discovered that vibrion butyrique (clostridium butyricum) lost it motility in a wet preparation under the microscope when it approached the edge of the preparation where it was exposed to the air. Pasteur used the name 'anaerobies' which was the origin of our word 'anaerobes'.

David J, Hentges et al demonstrated the broad classification of bacteria as anaerobic, aerobic or facultative based on the types of reaction they employed to generate energy for growth and other activities. In their metabolism of energy containing compounds, aerobes required molecular oxygen as a terminal electron acceptor and could not grow in its absence. Anaerobes, on the other hand, could not grow in the presence of oxygen. Oxygen was toxic for them and they therefore depended on other substances as electron acceptors. Their metabolism frequently was a

fermentative type, in which they reduced available organic compounds to various end products such as organic acids and alcohols. Understanding the general characteristics of anaerobiosis provided insight into how anaerobes could proliferate in damaged tissue and special care was needed in processing clinical specimens that might contain them. (1993) [19].

Holdeman, Cato, Moore et al showed in their study that the present systems for the classification of anaerobic bacteria have eliminated many of the problems of earlier systems. Anaerobic bacteria produced many different enzymes that were of importance in providing nutrients to the bacterial cells, as virulence factors, and in permitting organisms to colonize or survive under adverse conditions. (1979) [44].

Bieluch V M, Tally F P et al studied the interaction between aerobic and anaerobic organisms in abscess formation. The organisms presented, represented a subset of those normally found at nearby mucosal surfaces. Certain organisms most notably, Bacteroides fragilis emerged from the normal flora as an important organism in abscess formation. The contribution of both aerobic and anaerobic organisms in the formation of abscess must be remembered when one chooses antibiotic therapy for such infections (1983) [8].

Shapiro M E, Onderdonk A B, Kasper D L et al studied and showed that the polysaccharide capsule of Bacteroides fragilis was very important in the virulence of organism, which initiated cellular immune response to the capsular polysaccharide of Bacteroides fragilis (1982) [73].

Tzianabos A O, Onderdonk A B et al showed that the capsular polysaccharide complex of Bacteroides fragilis exhibit unusual biological properties. The capsular polysaccharide complex consisted two distinct polysaccharides, PS-A and PS-B. Analysis of these polysaccharides as well as of other charged polysaccharides of bacterial origin, before and after chemical modification, revealed that the oppositely charged groups were required for promotion of intraabdominal abscesses as well as for the distinct properties associated with the Bacteroides fragilis capsular polysaccharide complex and delineated one mechanism by which these biological responses occurred (1995) [86].

Meisel-Mikolajczyk F et al studied the antigenic structure of Bacteroides fragilis by immunochemical investigations. After chemical treatment 6 serologically active substances were obtained. The studied strain released serologically active substances into the culture medium. They also found that endotoxin was the bacterial fraction according to which the strain might be classified to the appropriate serotype (1980) [54].

Kasper D L, Hayes M E, Craft F O et al developed an indirect immunofluorescence assay for the isolation and identification of encapsulated strains of Bacteroides fragilis that was immunologically similar to those found in the reference strain of Bacteroides fragilis sub species fragilis. The capsular polysaccharide of Bacteroides fragilis sub species fragilis was a unique factor associated with the predominant subspecies Bacteroides fragilis isolated from clinical material (1977) [47].

Lindberg A A, Berthold P et al studied the presence of capsules in the strain of the Bacteroides fragilis group of bacteria isolated from clinical infections and from faecal flora using the Indian ink and ruthenium red staining methods. A total of 77% of the Bacteroides fragilis strains were encapsulated (1979) [51].

Polk B F, Bartlett J G et al studied and showed that the organisms of the genus Bacteroides represented the major group of obligate anaerobes involved in human infections. Bacteroides usually caused either bacteremia or localized abscesses. Of the numerous species of Bacteroides, Bacteroides fragilis was the single most frequent isolate. Furthermore, strains of Bacteroides fragilis had an immunologically common capsular polysaccharide. Those infected with Bacteroides fragilis developed antibodies to the capsular polysaccharide and these antibodies could be detected in a radioactive binding assay (1979) [65].

Patrick S, Stewart L D, Damani N et al studied and showed that a monospecific polyclonal antiserum, prepared against Bacteroides fragilis common polysaccharide antigen purified by polyacrylamide gel immunoblot detected Bacteroides fragilis. Bacteroides ovatus, Bacteroides thetaiotaomicron and Prevotella melaninogenica in pus samples from various anatomical sites by immunofluroscence microscopy of the pus. Of these, Bacteroides fragilis accounted for 33%. The results indicated that the common polysaccharide antigen, in contrast to the variable surface polysaccharide, was a suitable target for the immunodetection of Bacteroides fragilis in clinical samples from a range of anatomical sites (1995) [64].

T D Wilkins, D L Wagner et al showed that several variables affected the production of catalase by members of the "Bacteroides fragilis group" of anaerobic bacteria. Addition of hemin to media after autoclave sterilization, rather than before significantly increased production of catalase. Of the various media tested, the use of chopped meat broth resulted in the highest levels of catalase production. Of the various species, strains of Bacteroides fragilis and Bacteroides distasonis were catalase positive. A catalase well test, in which equal volumes of 3% hydrogen peroxide and chopped meat culture were mixed, was described and recommended for routine catalase tests (1978) [87].

Rissing J P, Buxton T B et al developed an indirect enzyme linked immunosorbent assay to detect specific Bacteroides fragilis antigens in the human urine specimens collected within 72 hours (1984) [68] .

Onderdonk A B, Kasper D L, Barlett J G et al in their studies showed the pathogenic potentials of encapsulated and unencapsulated strains of Bacteroides fragilis in animal models. They suggested that the abscess potentiating ability of encapsulated Bacteroides fragilis was related to the capsular polysaccharide, which appeared to potentiate abscess formation and might represent a virulence factor for this species (1977) [61].

Rotstein O D, Pruett T L, Simmons R L et al in their study showed that the surgical infections were almost always polymicrobial. True synergy existed between certain aerobes and anaerobes. Of the possible mechanisms of synergy; the most important seemed to be the ability of anaerobes, their metabolic products or their capsules to inhibit phagocytosis of aerobes by leucocytes (1985) [69].

Brook I et al showed that the Bacteroides fragilis group was one of the most important pathogens in polymicrobial infections. Bacteroides fragilis accounted for 63% of all the group isolates. The Bacteroides fragilis group resisted betalactam antibiotics by producing the enzyme betalactamase. The virulence of all members of the Bacteroides fragilis

group highlighted the need to direct antimicrobial therapy against all members of this group (1989) [10].

R P Karyakarte, B Deshmukh et al in their studies showed that with the introduction of the isolation techniques for obligatory anaerobes, attention was drawn towards the potential significance of anaerobic bacteremias. Anaerobiosis was produced by evacuation replacement systems using 10% carbondioxide and 90% hydrogen. The isolates were identified using a battery of biochemical reactions. Antimicrobial susceptibility testing was performed by the disc broth method. Among the anaerobes, Bacteroides fragilis was isolated from a patient with bacterial endocarditis. All the isolates were susceptible to metronidazole, chloramphenicol and tetracycline. They also showed that isolation of obligate anaerobes from blood might signal presence a serious underlying condition (1999) [48].

Rama Chaudary, Purvamathur et al demonstrated that the member of Bacteroides fragilis group were the most commonly isolated anaerobic pathogens in humans. Metronidazole was the drug of choice for preventing and treating such infections for 40 years. Although Bacteroides fragilis exhibited the broadest spectrum of recognized resistance to antimicrobial agents among anaerobes, the world wide rate of metronidazole resistance remained low. (<5%) (2001) [66] .

Brook I, Frazier E H et al studied over a period of 14 years (1973-1987), all the specimens submitted to the microbiology laboratory and demonstrated that Bacteroides fragilis accounted for 62% of total (1997) [11].

Chaudary R, T Majumdhar et al showed that, the genus Bacteroides was the most important anaerobic pathogens, commonly recovered from clinical infections. Different molecular approaches have redefined the genus Bacteroides. Rapid methods of isolation and identification of these groups of organisms have evolved to facilitate diagnosis. Development of antimicrobial resistance in Bacteroides was a disturbing issue. An understanding of the mechanisms of resistance would enable the designing of new antimicrobial agents for Bacteroides infection (1997) [15].

J E Sondag, M Ali et al studied the recovery of clinical anaerobic isolates on selective and non selective agar medium as well as time required to detect the isolates. The recovery of anaerobic isolates was higher when combination of media were used than when used alone. Also a total of 19% of anaerobes were detected after incubation for one day and 70% were detected after 2 days (1979) [76].

D L Draper, A L Barry et al described a simple screening test for separating Bacteroides fragilis from other anaerobic Gram negative

bacilli. This test utilized filter paper discs impregnated with 25 mg of oxgall, tested in conjunction with antibiotic identification discs. After 24 hours at 35^{O}C degree centigrade in a gaspak jar, resistance to kanamycin and bile was taken as a presumptive identification of Bacteroides fragilis (1977) [22].

Sapico F L, Canawati H N Witte J L et al studied the quantitative aerobic and anaerobic cultures of deep tissue biopsies performed on amputated infected lower limbs of 13 diabetic patients immediately after surgery and revealed that the most frequently isolated organisms were Enterococci, anaerobic Streptococci and species of Proteus, Clostridium and Bacteroides. They suggested that when antimicrobial therapy was indicated for these patients, the possibility of the concomitant presence of aerobes as well as anaerobes should be considered (1980) [72].

Louie T J, Barlett J G, Tally F P et al studied the aerobic and anaerobic bacteria in diabetic foot ulcers. They also suggested that when antimicrobial therapy was indicated, the selection of agents should consider the likelihood of a complex aerobic-anaerobic flora (1976) [52].

Catherine Amalia S, Calayco M D et al in their studies showed that patients who had chronic wounds or who had recently received antibiotic therapy might have been infected with Gram negative rods and those with foot ischemia or gangrene might have got obligate anaerobic pathogens,

which complicated diabetic foot infection and increased the risk of limb amputation and stump failure. The emergence of resistance anaerobic pathogens was increasing and this posed a problem in the choice of empiric antibiotic regimens (2002) [13].

De A, A Varaiya, M Mathur et al showed that anaerobic organisms were present in pleuropulmonary infections from empyema and pleural effusion (65.5% and 68.4% respectively). Amongest anaerobes, Gram negative anaerobes Prevotella species, Fusobacterium species, Bacteroides species predominated. They suggested antibiotic coverage for both aerobic and anaerobic in all cases of pleuropulmonary infections (2002) [20].

Sanderson P J, M Wren, A W Baldurn et al showed that Bacteroides species were the most common anaerobic organisms isolated from post operative wounds and from all operation sites except the lung (1979) [71].

Dunn D L, Simmons R L et al studied the exact role of anaerobic enteric flora in the pathogenesis and persistence of intraabdominal infections due to a perforation of a hollow viscus. This had been evaluated from the relatively few species that were found and compared with 100's of species populating the colon (1984) [23].

Louie et al studied that the infection in the diabetic foot was usually of polymicrobial etiology. The prevalence of anaerobic pathogens in the diabetic foot varied widely from 5% - 95%. The most frequent anaerobic isolate was Peptostreptococcus with other anaerobes like Bacteroides fragilis, Bacteroides melaninogenicus and Clostridia. All anaerobic isolates were subjected to susceptibility using E-test method. The only Bacteroides fragilis isolate subjected to susceptibility testing was resistant to metronidazole but sensitive to all the other antibiotics tested (1976) [52].

Douglas B, Vasey B et al studied and showed that the Bacteroides fragilis was a cause of residual abscess which had not been previously demonstrated. Special collection and culture methods were necessary to obtain positive cultures of this strictly anaerobic organism (1975) [21].

Elhag K M, Alwan M G et al showed the role of Bacteroides fragilis in the pathogenesis of acute appendicitis. Aerobic and anaerobic bacteria were isolated and members of Bacteroides fragilis group were the most common anaerobic isolates. He also suggested that it has a major role in the septic complication of acute appendicitis (1986) [25].

Das P, De A, Sharma et al studied and showed the predominant role of Anaerobic GPC and Bacteroides fragilis in the causation of brain abscess and subdural empyema (2000) [17].

Brook I, Frazier E G et al studied the microbiology of perirectal abscesses and illustrated the polymicrobial aerobic and anaerobic microbiology of perirectal abscesses in which the predominant anaerobe was Bacteroides fragilis (1997) [11].

Goldstein E J, B Wield et al studied the bacteriology of 224 human and animal bite wounds by culturing both aerobically and anaerobically. A total of 88 anaerobic strains were isolated, the most common being various Bacteriodes species (36 strains) (1978) [35].

England D M, J E Rosen Blatt et al studied both the aerobic and anaerobic cultures of bile from patients who underwent biliary tract surgery. Bacteroides fragilis was the single most commonly isolated anaerobe. This study demonstrated the frequent presence of anaerobes in patients with bactibilia and suggested that they be considered in the formulation of antimicrobial therapy for infections involving human biliary tracts (1977) [27].

Hedberg M, Nord C E et al studied the activity of old and newer antianerobic drugs against clinical isolates of Bacteroides fragilis group strains from different parts of Europe. Abdominal infections and wound were the most common sources of isolation and Bacteroides fragilis was the dominating species. Less than 1% was resistant to Imipenam, Piperacillin, Tazobactam, Metronidazole. 96% of the isolates were

betalactamase producers. They concluded that the antimicrobial resistance among Bacteroides fragilis group was increasing (2003) [40].

Williams and Wilkins et al showed that in their study that Clindamycin, Metronidazole and Chloramphenicol were three antimicrobial agents useful in the treatment of anaerobic infections. Metronidazole was effective in the treatment of infections involving Gram negative anaerobes, but it was unreliable in the treatment of Gram positive anaerobic infections and was ineffective in treating aerobic infections. Additionally Metronidazole was often the drug of choice in treating infections in which Bacteroides fragilis was a serious concern (2001) .

Shimada k, Inamtasu T, Sato K et al studied the antimicrobial activity of the recent clinical isolates like Bacteroides fragilis, Bacteroides thetaiotaomicron, Bacteroides vulgatus, determined by the agar dilution technique. These 3 species had similar susceptibility patterns. Metronidazole was active against all Bacteroides strains (2004) [74] .

Ellie J C, Goldstein and Dianne M Citron et al studied the invitro activities of Ofloxacin alone and in combinations with Metronidazole against anaerobic bacteria isolated from intraabdominal infections as determined by broth microdilution and showed that some Bacteroides

fragilis strains were susceptible and that most other Bacteroides fragilis strains were resistant to Ofloxacin (1991) [26].

Helstad A G, M A Hutchinson et al evaluated the aerobic thioglycollate broth disc and the vaspar overlay broth disc methods for the antibiotic susceptibility testing of anaerobes. Overall, the aerobic thioglycollate broth disc and vaspar overlay broth disc methods yielded agreements of 93.3% and 93% respectively with the NCCLS method (1984) [42].

P R Murray, A C Niles et al compared the results of anaerobic susceptibility tests performed with inocula prepared directly from agar isolation media and from overnight broth cultures. They concluded that the test organisms used in the testing grew better when the inoculum was prepared directly from the agar plates (1983) [58].

T D Wilkins, Lillian V et al developed a method for the determination of the antibiotic susceptibility of anaerobic bacteria by the use of a single disc diffusion technique and incorporation of the inoculum in pour plates. The method was standardized by correlation of zone diameters with minimal inhibitory concentrations determined in broths (1972) [90].

Kenneth K E, Deborah et al studied the invitro surveys of antimicrobial resistance among clinically important anaerobes and

showed that all the isolates of the Bacteroides fragilis group were susceptible to Piperacillin, Tazobactum and Metronidazole, while resistance to Imipenam and Meropenam was low (< 2%). They established and confirmed that clinically important anaerobes could vary widely in their antimicrobial susceptibilities (2001) [49] .

Y Yamashita, S Kohno, H Koga et al demonstrated the direct detection of Bacteroides fragilis from clinical specimens using the PCR method for amplifying a special fragment of the glutamine synthetase gene from Bacteroides fragilis. Using the nested PCR, they were able to detect as little as 1 bacterial cell or 100 fg of chromosomal DNA of Bacteorides fragilis and detected even in 2 culture negative samples (1994) [91].

Carvalho C B, Moreira J L et al in their study examined the epidemiological aspects and antimicrobial susceptibility profile of the Bacteroides fragilis group isolated from clinical and human intestinal specimens. The resistance rates and the variation in susceptibility patterns among the species isolated from clinical and human intestinal specimens emphasized the need for monitoring the susceptibility patterns of Bacteroides fragilis group organisms isolated (1996) [12].

Snydman D R, M c Dermott L et al in their study well documented the antimicrobial resistance, including plasmid – mediated resistance,

among Bacteroides fragilis group species. No documented resistance to either metronidazole (or) chloramphenicol was found. This study demonstrated that the rates of resistance of Bacteroides fragilis and non fragilis species of Bacteroides fragilis group were increasing (1996) [75].

Trinh S, Reysset G et al demonstrated that a PCR method was developed for detection of the nim genes encoding 5-nitroimidazole resistance in Bacteroides species. Two PCR primers specific for nim genes were designed, which allowed amplification of a 458bp fragment from all characterized plasmid and chromosome borne metronidazole resistance genes (1996) [85].

Stanley K, Lubbe M M et al in their study investigated the prevalence of Nim genes (proposed to encode a 5-nitroimidazole resistance product) in anaerobic / facultative anaerobic bacteria. DNA sequencing confirmed the presence of Nim A genes in Propionibacterium species, Actinomyces odontolyticus, Prevotella bivia and clostridium bifermentans and Nim B genes from Bacteroides fragils (1999) [79].

Stubbs S L, Brazier J S, Talbot P R et al developed a combined PCR – Restriction Fragment Length Polymorphism (RFLP) technique to characterize the 16s rRNA gene for identification purposes and the nitroimidazole resistance (nim) gene for detection of resistance to the major antimicrobial agent used to treat Bacteroides infections. PCR –

RELP of the nim gene products identified the 4 reported genes (nim A, B, C, D) and indicated the presence of a previously unreported nim gene in 5 strains. This novel nim gene exhibited 75% DNA sequence similarity with nim B (2000) [80].

Haggoud A, M Hand R A et al evaluated the dissemination of Nim genes, encoding 5-nitroimidazole resistance, among Bacteroides clinical strains isolated in Morocco using PCR. Although it was found that nim A and nim C genes were previously identified on Plasmids, he located them on chromosomes (2001) [37].

Edlund C, Hedberg M et al showed in their study that decreased uptake and altered reduction were believed to be responsible for metronidazole resistance. Five nim genes (A, B, C, D, E) had been identified in Bacteroides fragilis group species that conferred resistance to 5-nitroimidazole antibiotics. They added, that a knowledge of the status and the mechanisms of resistance helped in the selection of antimicrobial therapy and the design of new antimicrobial agents (2002) [24].

Jamal W Y, Rotimi V O et al in their study screened for infections caused by metronidazole resistant Bacteroides species and characterized the genes that encoded the metronidazole resistance and investigated by PCR and the PCR products were subjected to PCR – RFLP analysis.

They concluded that Nim genes, especially nim E and nim A genes mediated the drug resistance in these isolates (2004) [45].

Jeffrey, M Schapiro, Rachna Guptal et al in their study showed that the members of the Bacteroides fragilis group were among the most common anaerobic bacterial isolates in the clinical specimens. Metronidazole, a 5-nitroimidazole was often used as empirical therapy for anaerobic infections. They reported a case of Metronidazole resistant Bacteroides fragilis in Washington, demonstrating the presence of the **nim A** gene, encoding a nitroreductase previously shown to mediate resistance to 5-nitroimidazole antimicrobial agents in Bacteroides fragilis strains, by polymerase chain reaction and DNA sequencing using nim gene specific primers. Direct sequencing of the PCR product demonstrated 100% identity to the published nim A gene sequence (2004) [46].

Soki J, Gal M et al in their study investigated the constitution of nim gene types, their activating insertion sequence (IS) element, their localization (plasmid / chromosome) and Cfi A gene status in metronidazole resistant Bacteroides strains to examine their interchangeability. Southern hybridization and conjugative plasmid transfer were used to localize the nim E genes and plasmid functions. PCR was used to detect the IS elements and the Cfi – A genes. PCR

mapping was applied to detect the nim gene associated IS elements (2006) [78].

Sonja Lofmark, Hong Fang et al in their study found that nitroimidazole resistance (nim) genes were detected in 2% of clinical Bacteroides fragilis group strains isolated. In particular, Bacteroides fragilis was among the most clinically relevant anaerobic species. Metronidazole, a 5-nitroimidazole agent was particularly useful against Bacteroides species that tend to be resistant to a wide range of antimicrobial agents, although the rate of resistance remained low, at <1%, Metronidazole resistant organisms were beginning to emerge. The strains were screened for nim genes by PCR with specific primers and cleaved with restriction enzymes (2005) [77].

MATERIALS AND METHODS

This prospective study was conducted at Government Rajaji Hospital attached to Madurai Medical College. The period of the study was 8 months (November 2005 – June 2006). The study population consisted of 175 patients with varied infections admitted in different wards, Surgery, Surgical gastroenterology, Obstetrics and Gynaecology, Orthopaedics and Burns.

Selection of patients:

1. Post operative wound infections
2. Wound infections following burns
3. Diabetes mellitus patients with ulcers
4. Open injury following accidents
5. Intra abdominal abscesses
6. Decubitus ulcers
7. Septicaemia cases in Obstetrics and Gynaecology wards.

Collection and transportation of specimen:

Sterile swabs and sterile screw capped containers were used for sample collection. Liquid thioglycollate medium was used for both collection and transport of specimens for anaerobic incubation and glucose broth for aerobic organisms. Sterile cotton swabs were used for the wounds in post operative infections, burns, diabetic ulcers, decubitus

ulcers, open injury following accidents. The overlying and adjacent areas were prepared carefully with an antiseptic agent (70% isopropyl alcohol) to eliminate the surface organisms before specimens were obtained. Wounds were washed with sterile saline with all aseptic precautions and the swabs were taken from the depth of the wounds. When the discharge was minimal, the edges of the wound were squeezed to expel the contents. Three consecutive swabs were taken for each case on the same day.

Using sterile syringes, pus was aspirated from the diabetic ulcers, open injury, postoperative infections, decubitus ulcers, burns, after expelling all the air from the syringe and needle. Skin was disinfected with 70% isopropyl alcohol and pus aspirated by needle and syringe, in case of intra abdominal abscesses. From septicaemia cases, blood was drawn aseptically by venepuncture and immediately inoculated into the liquid transport medium at a ratio of 1 ml of blood to 10 ml of the medium, in two bottles, one with glucose broth for maximal recovery of strict aerobes and the other with thioglycollate broth for anaerobes.

With all aseptic precautions, under proper speculum visualization, pus and discharge was collected from cervix and vagina in cases of vaginal hysterectomy and puerperal infections.

Special precautions were taken to protect the specimens thus collected from the lethal effects of atmospheric oxygen right from the

collection till their incubation in the laboratory. All the clinical samples collected from the patients in sterile screw capped containers were transported to the Microbiology laboratory with proper labeling of name, age, sex, IP/OP No. and without any delay. In the laboratory, the samples in the liquid thioglycollate medium were incubated anaerobically for 24 – 48 hours, and the samples in the glucose broth were incubated aerobically for 24 hours. The liquid thioglycollate medium was boiled or steamed to drive off residual oxygen and was used on the day of steaming for anaerobic culture.

Processing of Specimens:

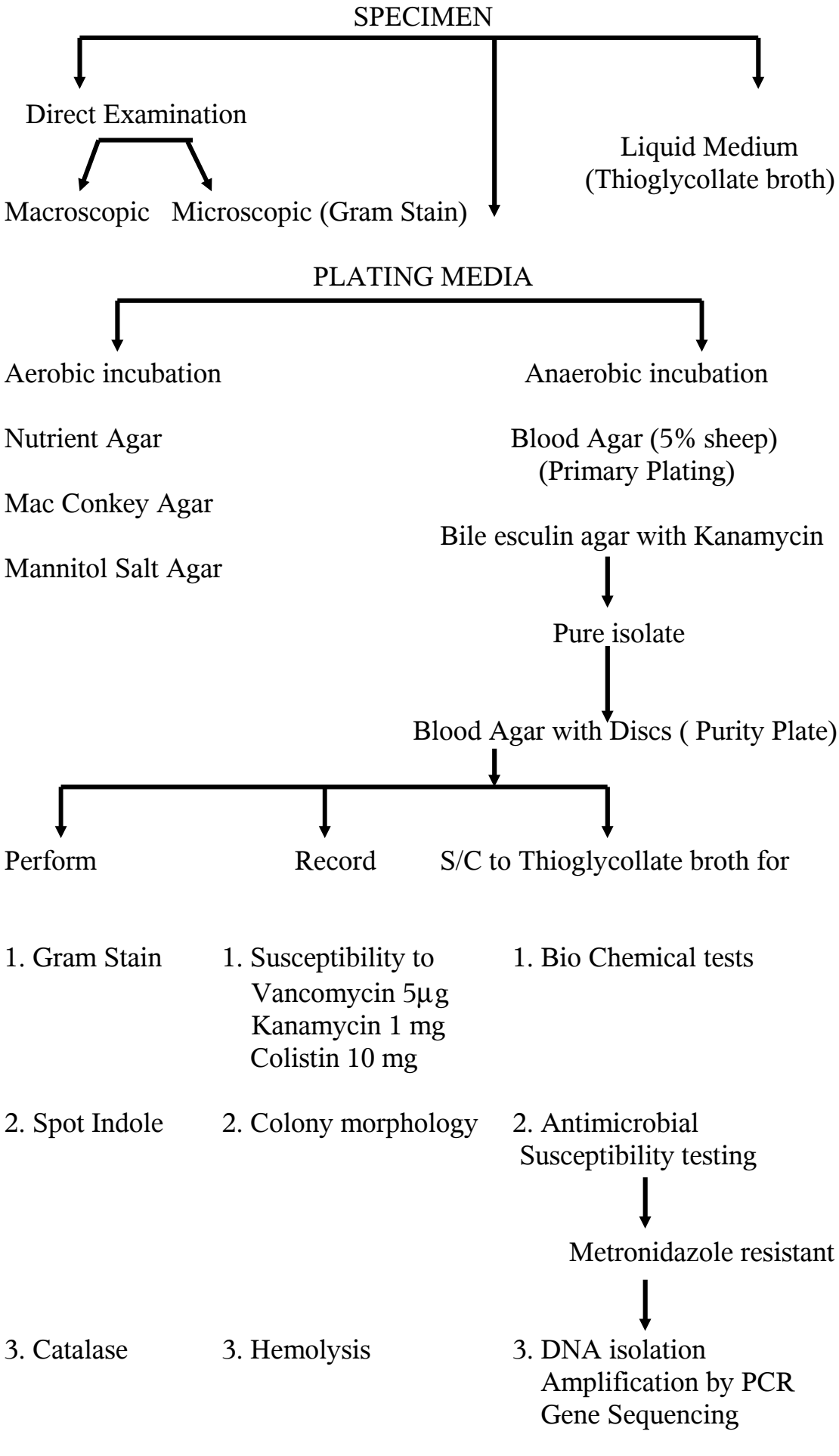
Among the three swabs, one swab was used for making a direct smear which was fixed by methanol. Gram staining was done and microscopic findings were noted. After 24 – 48 hours of incubation, both aerobic and anaerobic samples were inspected for turbidity, odour and purulence. A smear was made from the incubated specimens and Gram stained and microscopic findings were noted.

The other two swabs taken were one for anaerobic and other for aerobic culturing. The plates were selected according to the Gram's reaction. For aerobic Gram positive cocci, nutrient agar and 5% sheep blood agar were selected For aerobic Gram negative bacilli, nutrient agar, MacConkey agar and 5% sheep blood agar were selected. The samples under anaerobic incubation were streaked onto appropriate

plating media (5% sheep blood agar and bile esculin agar with kanamycin) and incubated in a Gas Pak anaerobic jar at 37°C for 48 hours.

After 48 hours of incubation the plates were examined for colony growth, morphology and hemolysis pattern. Specific colony of each type was selected, Gram stained and microscopic findings were noted. Colonies showing pale irregular staining, Gram negative bacilli were selected and streaked again onto 5%. Sheep blood agar plates for isolation of pure culture and also for special potency antibiotic disc susceptibility for the antibiotics Vancomycin, Colistin, Kanamycin. This was further incubated for 48 hours anaerobically at 37°C.

After 48 hours of incubation, colony morphology, hemolysis pattern, susceptibility to special potency antibiotic disc were recorded and Gram staining was done. Colonies showing Gram negative bacilli with pale irregular staining were subjected for spot Indole and catalase test and subcultured on to thioglycollate medium for further biochemical tests and antimicrobial susceptibility testing. All the metronidazole resistant isolates were selected and subjected for DNA isolation, which was amplified by PCR method and amplified product was subjected for gene sequencing.


SPECIMEN
Direct Examination
Macroscopic
Microscopic (Gram Stain)
Liquid Medium
(Thioglycollate broth)
PLATING MEDIA
Aerobic incubation
Anaerobic incubation
Nutrient Agar
Mac Conkey Agar
Mannitol Salt Agar
Blood Agar (5% sheep)
(Primary Plating)
Bile esculin agar with Kanamycin
Pure isolate
Blood Agar with Discs (Purity Plate)
Perform
Record
S/C to Thioglycollate broth for
1. Gram Stain
2. Spot Indole
3. Catalase
1. Susceptibility to
Vancomycin 5µg
Kanamycin 1 mg
Colistin 10 mg
2. Colony morphology
3. Hemolysis
1. Bio Chemical tests
2. Antimicrobial
Susceptibility testing
Metronidazole resistant
3. DNA isolation
Amplification by PCR
Gene Sequencing

Microscopic Examination:

Smears were methanol fixed and were Gram stained and basic fuschin was used as the counter stain, left for 5 minutes and microscopic findings were noted.

It showed pleomorphic, Gram negative coccobacillary forms with pale irregular staining.

Culture:

A combination of enriched, selective, non selective plating media were used for the primary isolation and presumptive identification of obligate anaerobes from the clinical material. Freshly prepared media were used. 5% sheep blood agar plates were used for the isolation of all bacteria. Bile esculin agar with Kanamycin was used for the selective isolation and presumptive identification of Bacteriodes fragilis.

Liquid thioglycollate medium was used as a backup culture. The upper 1/3 of the fluid medium was pink in color. It required steaming at $100^{O}C$ for few minutes to restore anaerobic conditions and it was used on the day of steaming. Under the laminar airflow, the processing was carried out aseptically.

Direct plating was done in the blood agar and bile esculin agar with kanamycin plating media and incubated in a Gaspak anaerobic jar for 48 hours at 37 ^{O}C.

Gaspak Anaerobic Jar:

This consisted of a transparent unbreakable polycarbonate jar of 1.5 litres capacity, with a sturdy aluminium lid, a metal clamp with a 1 nitrile rubber sealing O ring. A stainless steel petriplate carrier which had 2 clips, one for anaerogas packs and the other for anaero indicator tablet.

Anaerogas packs:

This is a disposable oxygen absorbing and carbondioxide generating agent for use in anaerobic jars. No catalyst or pressure gauge is necessary for this pact, since no gas pressure is generated.

Anaeroindicator tablet:

This tablet was kept intact with the transparent cover. On removal, the original color may change to purplish blue due to the absorption of oxygen. Under anaerobic conditions this color will change to pink.

Principle:

The Gaspak anaerobic jar utilized the commercially available hydrogen and carbondioxide generator envelope. Production of heat within a few minutes and subsequent development of moisture on the envelope and the walls of the jar were indications that the catalyst and generator envelope were functioning properly. The reduced conditions were achieved in 1-2 hrs. It took few hours for the tablet to turn completely pink to indicate anaerobic conditions inside the jar.

At the same time the specimens were inoculated onto appropriate aerobic plating media and incubated for 24 hours at $37^{O}C$.

After 48 hours of anaerobic incubation, primary plates were examined and the colony growth, morphology, haemolysis pattern and pigmentation were recorded.

Standardisation of the Jar:

The jar was standardized using a strict aerobe and an obligate anaerobe. Pseudomonas aeruginosa was streaked onto a blood agar plate and kept in the jar. A swab from the environment was incubated onto thioglycollate medium, incubated for two days and then plated onto blood agar plate and incubated anaerobically in the gaspak jar for 48 to 72 hours along with pseudomonas. It was found that Pseudomonas failed to grow under anaerobic conditions. The specimens from the environmental sampling produced growth under anaerobic conditions.

The inoculated Petriplates were placed in the carrier, one himedia's anaeroindicator tablet pack was removed from the sachet and inserted into the upper clip on the petriplate carrier immediately. Now the plate carrier was lowered into the polycarbonate base. Then the top of one required anaerogaspack was cut, and sachet removed and placed in the lower clip of the carrier plate. The jar was tightly closed and clamped. Knurled wheel was screwed down until tight.

Blood agar plate:

Grey white, glistening, non hemolytic, 1-3 mm diameter colonies were selected for grams staining, which showed pale, irregularly staining, Gram negative pleomorphic rods.

Bile esculin agar with Kanamycin:

Grey, circular, entire, raised colonies with stippling in the medium around the colonies. These colonies indicated that they were bile resistant and able to grow in the presence of bile and Kanamycin and hydrolyse esculin. These colonies were subjected for Grams staining. With the above colony morphology and growth pattern, it was concluded to be bile resistant Bacteroides species. All the isolates which were considered as Bacteroides species were sub cultured onto blood agar plate (freshly prepared) for isolation in pure culture.

Single colony of each distinct type was sub cultured onto 5% sheep blood agar plate

- The blood agar plate was divided into 2 quadrants, one for lawn culture and the other for streaking. First quadrant was streaked back and forth several times to ensure an even lawn of heavy growth in which the following special potency antibiotic discs, Kanamycin 1mg, Vancomycin 5µg and colistin 10mg were placed, for the preliminary grouping of anaerobes, and this had no

implication on the susceptibility of an organism for antibiotic therapy. A zone of inhibition of 10mm or less indicated resistance.

- The second quadrant was streaked for the isolation of organism in pure culture, and incubated in Gaspak anaerobic jar for 24-48 hours at 37°C. After 48 hours of anaerobic incubation, colony morphology, hemolysis pattern, susceptibility to special potency antibiotic disks were recorded.

PURE CULTURE BLOOD AGAR WITH DISCS

PERFORM	RECORD	SUBCULTURE TO THIOGLYCOLLATE BROTH FOR
1. Gram stain	1. Susceptibility to Vancomycin (5µg)	1. Biochemical tests
2. Spot Indole	Kanamycin (1mg)	2. Antimicrobial Susceptibility testing
	Colistin (10mg)	
3. Catalase	2. Colony morphology 3. Hemolysis pattern 4. Pigment	

Special potency antibiotic disc:

This was added as an aid in determining the Gram reaction and in separating Bacteroides and Fusobacterium species. The zones of inhibition were measured. Results were interpreted as sensitive or resistant. The isolates were resistant to all the three special potency antibiotic discs.

Blood agar plate:

Grey white, glistening, nonhemolytic colonies were selected for G Gram staining.

Pale irregular staining, Gram negative, pleomorphic rods, resistant to all 3 special potency antibiotic discs were considered Bacteroides fragilis group and were subjected to the following tests.

1. **Spot Indole test:**
    - ❖ The Indole end product of the action of tryptophanase on tryptophan can be detected by its ability to combine with certain aldehydes to form a coloured compound.
    - ❖ This test demonstrated the production of Indole by splitting tryptophane.
    - ❖ A disc impregnated with 1% para-dimethyl amino cinnamaldehyde in 10% Hydrochloric acid was placed on a petridish and the colony to be tested was streaked on to the disc.
    - ❖ Rapid development of the blue color indicated positive reaction.
    - ❖ Bacteroides fragilis are spot Indole negative.

Catalase test:

The breakdown of H_2O_2 into O_2 and H_2O is mediated by the enzyme catalase. When a small amount of the organism that produces catalase was introduced into H_2O_2, rapid elaboration of bubbles of O_2, the gaseous product of the enzyme activity will be produced.

* Freshly prepared 15% H_2O_2 was taken in a small test tube.
* Using a plastic, disposable inoculating loop (or) wooden applicator stick, some of the growth from the purity plate was removed.
* Production of prompt effervescence indicated catalase positive.
* Bacteroides fragilis were catalase positive

The colonies which were spot Indole negative and catalase positive were further tested for fermentation of sugars for confirmation of species.

Fermentation of sugars:

Tubes of peptone yeast broth containing various carbohydrates such as glucose, maltose, sucrose, lactose, arabinose and mannitol were inoculated. Uninoculated tubes form each batch of medium were incubated along with inoculated tubes. Glucose (with acid no gas), maltose, sucrose, lactose were fermented, but arabinose and mannitol were not fermented.

Only Bacteroides fragilis were sucrose positive and arabinose negative. With the above mentioned colony morphology, hemolysis, resistance to bile and 3 special potency antibiotic discs, spot Indole negative and catalase positive were considered as Bacteroides fragilis and subjected for antimicrobial susceptibility testing for anaerobes.

Procedure for Antimicrobial susceptibility testing (Broth Disc Test):

The broth disc test described by Kurynski & co workers was followed. The following antimicrobial discs were added to the thioglycollate medium as follows in screw capped tubes and incubated the tests under the anaerobic conditions.

(TABLE 5 –5 OF WADSWORTH ANAEROBIC BACTERIOLOGY MANUAL)

PREPARATION OF BROTH DISC TUBES

Sl. No.	Drugs	Disc Content	No. of Discs 5ml Medium	Test Concentration/ml
1	Carbenicillin	100	3	60
2	Cefoperazone	75	2	30
3	Chloramphenicol	30	3	18
4	Clindamycin	2	4	1.6
5	Metronidazole	80	1	16
6	Penicillin G	10	1	2
7	Cefotaxime	30	3	18
8	Tetracycline	5	3	3

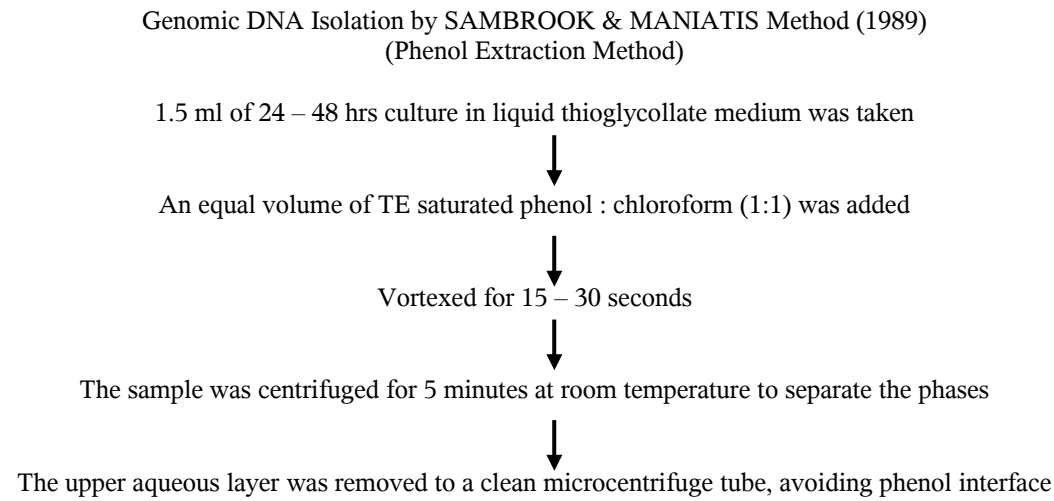
Tubes were inoculated with 0.1 ml of 24 -48 hours broth culture. 1 tube of the medium without antimicrobial served as a growth control. After inoculation, the tubes were gently inverted 2 or 3 times to ensure

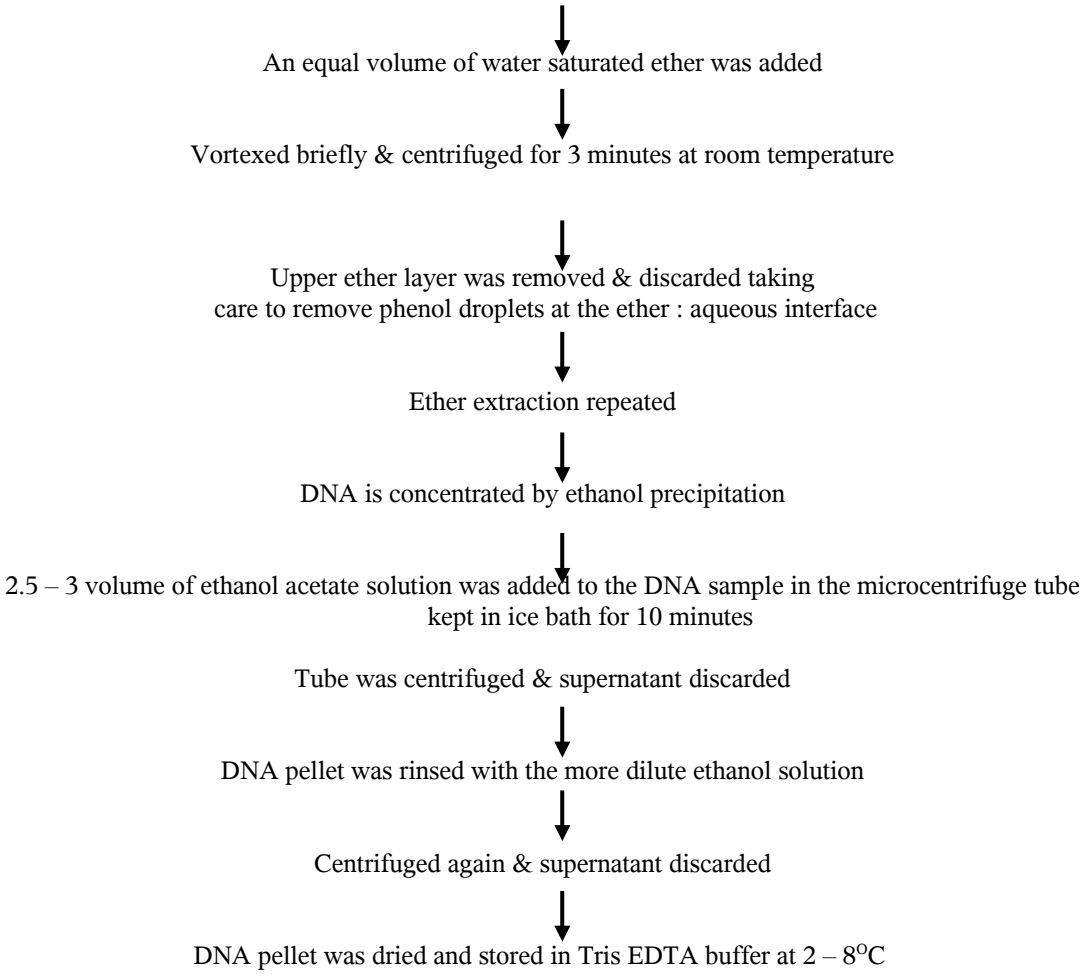
adequate mixing of antimicrobial and inoculums. The tubes were incubated for 18 – 24 hours anaerobically. Susceptibility of the drugs were identified by the absence of growth or less than 50% of the growth as compared to the control tube. The sensitivity and resistance of the Bacteroides fragilis to the antimicrobials were determined by comparing the tests MIC (Minimum Inhibitory Concentration) range values with the accepted MIC range values.

Due to technical constraints, it was proposed to send metronidazole resistant Bacteroides fragilis to an International Centre for gene sequencing.

DNA ISOLATION

For the isolation of genomic DNA, from Bacteroides fragilis, the organism was subcultured and grown in liquid thioglycollate medium anaerobically (24-48 hours/37^{O}C). The samples were taken and the DNA isolation was carried out at the Department of Immunology, The American College, Madurai.





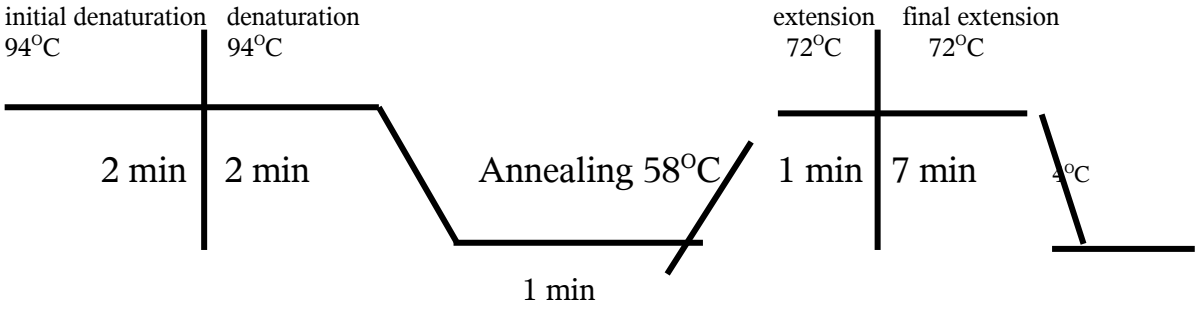
Polymerase Chain Reaction (PCR)

The PCR was carried out at Cancer Biology, School of Biological Science, Madurai Kamaraj University in the following manner. The amplification of isolated DNA fragments using the PCR was performed in the Applied Biosystem Gene (ABG) Amp PCR 2700 by adding the following reagents to a 1.5ml tube, respectively:

Reagents	Quantity
Template Genomic DNA molecule	10µl
2 Primers (Forward & Reverse)	10µl
Nucleotides	2µl
Buffer	10µl
Taq DNA polymerase	3µl
$MgCl_2$	6µl

The cycling protocol consisted of 35 cycles of 3 temperatures : strand denaturation at 94^{O}C, primer annealing at 58 ^{O}C and primer extension at 72 ^{O}C. After PCR, aliquots of the mixture are loaded onto an agarose gel and electrophoresed to detect amplified product for further sequence analysis.

(35 Cycles)



Sequence Analysis:

Short sequencing i.e. upto 600 bp of the amplified DNA was done commercially at MICROSYNTH LABORATORY, SWITZERLAND. The 16S rDNA homology analysis for metronidazole sensitive and resistant strains of Bacteroides fragilis was done using NCBI BLAST.

RESULTS

A total of 175 samples from various types of infection were collected, 67 (38.2%) from General Surgical, 12 (6.9%) from Surgical Gastro enterology, 29 (16.6%) from Burns, 33 (18.9%) from Orthopaedics and 34 (19.4%) from Obstetrics & Gynaecology wards of Government Rajaji Hospital, Madurai. **Thus the infections were more common in General Surgical ward.** This is shown in Table No. 1.

TABLE 1

WARDWISE DISTRIBUTION OF CASES

Sl. No.	Wards	Number of Cases
1	**General Surgery**	**67 (38.2%)**
2	Surgical Gastroenterology	12 (6.9%)
3	Burns	29 (16.6%)
4	Orthopaedics	33 (18.9%)
5	Obstetrics & Gynaecology	34 (19.4%)
TOTAL		175 (100%)

The 175 samples were further analysed according to the clinical conditions and it was found that 66(37.8%) were from post operative wound infections, 42 (24%) samples from diabetic foot ulcers, 29 (16.6%) samples from wound infections following burns, 27 (15.4%) samples from open injury following accidents, 4 (2.2%) from septicemia cases and 4 (2.2%) samples from decubitus ulcers, 3(1.7%) samples from intra abdominal abscesses. Distribution of samples according to the clinical conditions showed **more number of samples were collected**

from the post operative wound infections followed by diabetic foot ulcers. This is given in Table No. 2.

TABLE 2

DISTRIBUTION OF CASES Vs CLINICAL CONDITIONS

Sl. No.	Clinical Conditions	Number of Isolates
1	**Post Operative wound infections**	**66 (37.8%)**
2	Wound infections following burns	29 (16.6%)
3	Diabetic foot ulcers	42 (24%)
4	Open injury following accidents	27 (15.4%)
5	Decubitus ulcers	4 (2.2%)
6	Septicaemia	4 (2.2%)
7	Intraabdominal abscesses	3 (1.8%)

The total 175 samples collected were analysed to know the distribution of cases specimen wise. 133 (76%) samples were from pus, 20 (11.4%) samples were collected from blood, 22 (12.6%) samples were collected from tissue aspirates. And it was found that **more number of samples were collected from pus**. This is shown in Table No. 3

TABLE 3

SPECIMEN WISE DISTRIBUTION OF CASES

Sl.No.	Specimens	Number of Isolates
1	**Pus**	**133 (76%)**
2	Blood	20 (11.4%)
3	Aspirate	22 (12.6%)
TOTAL		175 (100%)

All the samples collected were further analysed for the ward wise distribution of cases and it was found that out of the 133 (76%) samples

from pus, 70 (52.6%) were from General Surgery, 14 (10.6%) were from Surgical Gastroenterology, 17 (12.8%) were from Burns, 21 (15.8%) from Orthopaedics and 11 (8.2%) from Obstetrics and Gynaecology wards. Out of the 20 blood samples collected, 6 (30%) were from General Surgery, 2 (10%) were from Surgical Gastroenterology, 4 (20%) were from Burns, 2 (10%) from Orthopaedics and 6 (30%) from Obstetrics and Gynaecology wards. Of the total 22 (12.6%) aspirates, 11 (50%) were collected from General Surgery, 4 (18.1%) from Surgical Gastroenterology, 2 (9%) from Burns, 3(13.6%) from Orthopaedics, 2 (9%) from Obstetrics and Gynaecology wards. **It was found that more number of pus samples (52.6%), aspirates (50%) were collected from the General Surgery ward.** This is shown in Table No. 4

TABLE 4

WARD WISE DISTRIBUTION OF SPECIMEN

Sl. No.	WARDS	Pus		Aspirate		Blood	
		Total	%	Total	%	Total	%
1	**General Surgery**	**70**	**52.6**	11	50	6	30
2	Surgical Gastroenterology	14	10.6	4	18.1	2	10
3	Burns	17	12.8	2	9	4	20
4	Orthopaedics	21	15.8	3	13.6	2	10
5	Obstetrics & Gynaecology	11	8.2	2	9	6	30
TOTAL		133	75.4	22	13.1	20	11.4

The total 175 samples were analysed for sex wise distribution of cases and it was found that 93 (53.1%) samples were collected from male patients, 82 (46.9%) samples from female patients. The age wise distribution of cases were also analysed and it was found that **11.4%**

were collected from males in the age group 36 – 45 years, 12% were collected from females in the age group 26 – 35 years. This is shown in Table No. 5

TABLE 5

AGE / SEX WISE DISTRIBUTION OF CASES

Sl. No.	Agewise Distribution	Male	Female
1	0 – 15 years	1 (0.5%)	1 (0.5%)
2	16 – 25 years	17 (9.7%)	16 (9.1%)
3	**26 – 35 years**	18 (10.2%)	**21 (12%)**
4	**36 – 45 years**	**20 (11.4%)**	16 (9.1%)
5	46 – 55 years	12 (6.8%)	16 (9.1%)
6	56 – 65 years	15 (8.5%)	6 (3.4%)
7	> 65 years	10 (5.7%)	6 (3.4%)
Total		93 (53.1%)	82 (46.9%)

All the clinical samples collected were analysed based on the primary Gram straining reaction. Out of the 175 samples, 149 (85.1%) samples were Gram negative and 26 (14.9%) samples were Gram positive. Thus it was found that **there were more number of Gram negative isolates** than Gram positive isolates.

The Gram differentiated samples were further analysed for the presence of aerobes and anaerobes and it was found that, out of the 149 (85.1%) Gram negative isolates, 117 (78.6%) were aerobes and 32 (21.4%) were anaerobes and all the Gram positive isolates were aerobes. Thus **Gram negative aerobes were commonly found in the clinical isolates**, then

the Gram negative anaerobes and then the Gram positive aerobes. There were no Gram positive anaerobes. This is also shown in Table No. 6

TABLE 6

AEROBES, ANAEROBES Vs PRIMARY GRAM REACTION

Sl. No.	GRAM REACTION		Number of Isolates
1	**GRAM NEGATIVE (149) (85.1%)**	**Aerobes**	**117 (78.6%)**
		Anaerobes	32 (21.4%)
	Total		149 (85.1%)
2	GRAM POSITIVE (26) (14.9%)	Aerobes	26 (100%)
		Anaerobes	0 (0%)
	Total		26 (100%)

Out of the 175 samples, the ward wise distribution of both aerobes and anaerobes were compared and analysed which showed that 27.4% aerobes and 10.2% anaerobes were from General Surgery, 15.4% aerobes and 2.8% anaerobes were from Burns, 16% aerobes and 1.7% anaerobes were from Orthopaedics, 16.5% aerobes and 1.7% anaerobes were from Obstetrics & Gynaecology and 6.2% aerobes and 1.7% anaerobes were from Surgical Gastroenterology wards. Thus it was found that **both aerobes and anaerobes were isolated from all the wards and 10.2% anaerobic isolates were from General Surgery ward.** This is shown in Table No. 7

TABLE 7

WARD WISE DISTRIBUTION AND COMPARISON OF AEROBES AND ANAEROBES

Sl. No.	NAME OF THE WARD	AEROBES	ANAEROBES
1	**General Surgery**	**48 (27.4%)**	**18 (10.2%)**
2	Burns	27 (15.4%)	5 (2.8%)
3	Orthopaedics	28 (16%)	3 (1.7%)
4	Obstetrics and Gynaecology	29(16.5%)	3 (1.7%)
5	Surgical Gastroenterology	11 (6.2%)	3 (1.7%)
	Total	143 (81.7%)	32 (18.3%)

As Bacteroides fragilis is the common Gram negative anaerobe from wound infections, all the 32 Gram negative anaerobes were analysed ward wise and it was found that out of the total 32 (18.2%) Bacteroides fragilis isolated, 18 (10.2%) were from General Surgery ward, 5 (2.8%) were isolated from Burns ward, 3 (1.7%) were isolated from Orthopaedics, Surgical Gastroenterology and Obstetrics & Gynaecology wards each. Thus it was found that **more number of Bacteroides fragilis were isolate from General Surgery wards (10.2%)** then the Burns ward (2.8%) and all the other wards showed less number of isolates. This is shown in Table No. 8.

TABLE 8

WARD WISE DISTRIBUTION OF BACTEROIDES FRAGILIS

Sl. No.	Name of the Ward	Number of Isolates of Bacteroides fragilis
1	**General Surgery**	**18 (10.2%)**
2	Burns	5 (2.8%)
3	Orthopaedics	3 (1.7%)
4	Obstetrics and Gynaecology	3 (1.7%)
5	Surgical Gastroenterology	3 (1.7%)
Total		32 (18.2%)

Bacteroides fragilis isolates were further analysed according to the clinical conditions and it was found that out of the total 32 isolates, 14 (8%) were isolated from post operative wound infections, 11 (6.2%) isolated from diabetic foot ulcers, 2 (1.1%) isolates were from wound infections following Burns, 2 (1.1%) were from open injury following accidents, 1 (0.5%) was isolated from decubitus ulcers, septicaemia and intraabdominal abscess each. It was found that **more number of isolates were from post operative wound infections (8%)** then the diabetic foot ulcers (6.2%), equal number of isolates from Burns and open injury following accidents and least number from other infections. This is shown in Table No. 9.

TABLE 9

DISTRIBUTION OF BACTEROIDES FRAGILIS Vs CLINICAL CONDITIONS

Sl. No.	Clinical Conditions	Number of Isolates
1	**Post Operative wound infections**	**14 (8%)**
2	Wound infections following burns	2 (1.1%)
3	Diabetic foot ulcers	11 (6.2%)
4	Open injury following accidents	2 (1.1%)
5	Decubitus ulcers	1 (0.5%)
6	Septicaemia	1 (0.5%)
7	Intraabdominal abscesses	1 (0.5%)
Total		32 (18.2%)

All the 32 Bacteroides fragilis were further analysed to study the age / sex wise distribution of the isolates. It showed that out of the 32 isolates, 19 (10.8%) were isolated from male patients and 13 (7.4%) were isolated from female patients. Among the male patients, 2.85% isolates were in the age group 46 – 55 years and in the female patients, 2.2% isolates were in the age group 26 – 35 years. This is shown in Table No. 10.

TABLE 10

AGE / SEX DISTRIBUTION OF BACTEROIDES FRAGILIS

Sl. No.	Agewise Distribution	Male	Female
1	0 – 15 years	1 (0.5%)	-
2	16 – 25 years	3 (1.7%)	2 (1.1%)
3	**26 – 35 years**	2 (1.1%)	**4 (2.2%)**
4	36 – 45 years	3 (1.7%)	3 (1.7%)
5	**46 – 55 years**	**5 (2.8%)**	2 (1.1%)
6	56 – 65 years	3 (1.7)	1 (0.5%)
7	> 65 years	2 (1.1%)	1 (0.5%)
Total		19 (10.8%)	13 (7.4%)

Further, the 32 Bacteroides fragilis isolates were analysed according to the specimens and it was found that out of the total 32 Bacteroides fragilis, 28 (21%) were isolated from pus, 2 (10%) were isolated from blood and 2 (9%) were isolated from aspirates. **Thus maximum recovery of Bacteroides fragilis isolates were from pus (21%),** and less number of isolates were from blood and aspirates. This is shown in Table No. 11.

TABLE 11

SPECIMEN WISE DISTRIBUTION OF BACTEROIDES FRAGILIS

Sl. No.	Specimens	Number of Isolates
1	**Pus**	**28 (21%)**
2	Blood	2(10%)
3	Aspirate	2 (9%)
TOTAL		32 (18.2%)

All the 32 specimens were further analysed for the ward wise distribution and it was found that out of the total 28 (21%) pus, 14 (10.5%) were from General Surgery, 3 (2.2%) from Surgical Gastroenterology, 4 (3%) from Burns, 5 (3.8%) from Orthopaedics and 2 (1.5%) Obstetrics and Gynaecology ward. Out of the 2 (9%) aspirates collected, 1 (4.5%) was collected from General Surgery and Burns ward each. Out of the 2 (10%) blood samples collected, 1 (5%) was collected from Burns and Obstetrics and Gynaecology ward each. This is shown in Table No. 12

TABLE 12

WARD WISE DISTRIBUTION OF SPECIMEN

Sl. No.	WARDS	Pus		Aspirate		Blood	
		Total	%	Total	%	Total	%
1	**General Surgery**	**14**	**10.5**	1	4.5	-	-
2	Surgical Gastroenterology	3	2.2	-	-	-	-
3	Burns	4	3.0	1	4.5	1	5
4	Orthopaedics	5	3.8	-	-	-	-
5	Obstetrics & Gynaecology	2	1.5	-	-	1	5
TOTAL		28	21.0	2	9.0	2	10.0

All the 32 Bacteroides fragilis isolates were further analysed for the susceptibility to antimicrobials including metronidazole and it was found that out of the 32 isolates, 28 (87.5%) were susceptible & 4 (12.5%) were resistant for carbenicillin, 29 (90.7%) susceptible and 3 (9.3%) resistant for cefaperazone, 31 (96.9%) sensitive and 1 (3.1%) resistant for chloramphenicol, 28 (87.5%) sensitive and 4 (12.5%) resistant for clindamycin, 31 (96.9%) sensitive and 1 (3.1%) resistant for metronidazole and 26 (81.2%) susceptible and 6 (18.8%) resistant for penicillin, 30 (93.8%) susceptible and 2 (6.2%) resistant for cefotaxaine, 29 (90.7%) susceptible and 3 (9.3%) resistant for tetracycline. Thus it was found that **only one organism was resistant for metronidazole and chloramphenicol i.e. 3.1% each,** which was also sensitive to clindamycin and all the other antimicrobials showed resistance more than 6%. This is shown in Table No. 13.

TABLE 13

ANTIMICROBIAL SUSCEPTIBILITY TESTING

Sl.No.	Drugs	Total Number Sensitive (%)	Total Number Resistant (%)
1	Carbenicillin	28 (87.5%)	4 (12.5%)
2	Cefaperazone	29 (90.7%)	3 (9.3%)
3	Chloramphenicol	31 (96.9%)	1 (3.1%)
4	Clindamycin	28 (87.5%)	4 (12.5%)
5	**Metronidazole**	31 (96.9%)	**1 (3.1%)**
6	Penicillin G	26 (81.2%)	6 (18.8%)
7	Cefotaxime	30 (93.8%)	2 (6.2%)
8	Tetracycline	29 (90.7%)	3 (9.3%)

It was found that the only metronidazole resistant Bacteroides fragilis was isolated from the General Surgery ward. The DNA of this only metronidazole resistant Bacteroides fragilis isolate and 1 sample of metronidazole sensitive Bacteroides fragilis isolate were sent for sequence analysis. The DNA of the metronidazole resistant Bacteroides fragilis and the one metronidazole sensitive Bacteroides fragilis were amplified by PCR and the amplified DNA was subjected for DNA short sequencing at MICROSYNTH LABORATORY, SWITZERLAND.

The sequencing data were analysed by comparison of 16S rDNA sequences with the Gen Bank sequence by using the Basic Local Alignment Search tool (BLAST). The sequence is given as follows:

Metronidazole sensitive Bacteroides fragilis 16s rDNA sequence

```
agagtttgat cctngctcag gatnaacgct agctacaggc ttaacacatg caagtcgagg
ggcatcagga agaaagcttg ctttctttgc tggngaccgg cgcacgggtg agtaacacgt
atccaaccng ccctttactc gggnatagcc tttcgaaaga aagattaata cccgatagca
taatgatncc gcatggtttc attantaaag gattcnggta aaggatgggg atncgttcca
ttaggttgtt ggngaggtaa cggcccacca agccttngat ggataggggt tctgagagga
```

aggtccccna cattggaact gagacacggt ccaaactcnt acgggaggca gcaatcgaat tncg

Metronidazole resistant Bacteroides fragilis 16s rDNA sequence

agagtttgan cctngctcac gattaacgct agctacaggc ttaacacatg caagtcgagg ggcatcagga agaaagcttg ctttctttgc tggcgaccgg cgcacgggtg agtaacaggt atccaaccng cccttnactc gggnatagtc tttcgaaaga aagattaata cccgatagca taatnatncc gcatggtatc attantaaag gattcnggta aaggatgggg atncgttcca ttaggttgtt ggngaggtag cggcncacca agtcttngat ggataggggt tctgagagga aggtccccna cattggaagt gagncacggt ccaaactcnt atgggaggca gcaatcgaat tncg

The 16S rDNA homology analysis was done using NCBI BLAST. It showed approximately 99% homology to Bacteroides fragilis genome. This establishes beyond doubt the biochemically proved strain in indeed *Bacteroides fragilis*

The homology analysis between the sensitive and resistance strains showed a difference of only 2.4% between them establishing them as separate strains.

DISCUSSION

Anaerobes are increasingly recognized as important pathogens in post operative wound infections, deep seated abscesses, burns etc., Metronidazole is the drug of choice in treating anaerobic infections. Bacteroides fragilis is one important anaerobe in which there is emerging resistance to metronidazole. Hence this study on **Bacteroides Fragilis in Sepsis – Microbiological studies** was aimed at finding the Gram negative anaerobe, Bacteroides fragilis which are metronidazole resistant and to find the gene sequencing of the resistant strain. For the isolation of the Bacteroides fragilis, cases of sepsis from various wards at Government Rajaji Hospital, Madurai were studied and the highest number of the cases of sepsis were found in the General 0Surgical ward (38.2%) where the post operative wound infection was the most essential problem. **Anielski et al** [2] in their study on wound infection, also revealed that the post operative wound infection in Surgical ward was very common due to longer pre operative stay in the hospital, duration of operation longer than 1 hour, emergency mode of operation, contaminated, dirty infective operations in traditional wound classification system and open drainage. This is in support of our selection of cases where the maximum number of cases were collected in General Surgical ward.

In the present study it was found that 53.1% of the cases selected were males and 46.9% were females. Thus the infection rate was found to be more common in males than females. Similar study by **Anielski et al** [2] who had proved the predominance of males 57.6% rather than females 42.4% in their wound infection, is in support of our study. **Nicolle L E et al** [60] explained the risk factor for the wound infection to be the functional status and behaviour status like smoking which are common in males. This may be responsible for more number of cases of males in the present study.

In the present study it was also found that in the males, the infection rate was common in the age group 36 – 45 years (11.4%) whereas in the females the age group was 26 – 35 years (12%). **Anielski et al** [2] have demonstrated the mean age of wound infection in both sexes to be 40.9 years. **Shapiro M et al** [73] demonstrated younger age in females especially after hysterectomies. These are in support of the present study, the cause for the younger age in females may be due to increased incidence of pelvic infection during active reproductive age group of 26 – 35 years, whereas in the males, the higher age group may be attributed to metabolic derangement, functional status and behaviour [60] .

In the present study, the common specimen collected for the analysis of Bacteroides fragilis were pus (76%), the wound aspirate (12.6%) and the blood (11.4%). Most of the pus samples were from the deep abscesses and aspirates were from the deep wounds and blood were from the septic cases. The study by **Cruse P et al** [16] about wound infection surveillance demonstrated that most of the anaerobic species of organisms were isolated from abscesses and were mostly polymicrobial. Among the anaerobes, Bacteroides species were common. Similarly **Alridge et al** [1] also demonstrated anaerobic bacteria which were polymicrobial mostly from Surgical interventions and they proved drainage of an abscess and debridement of devitalized tissue was the primary treatment. These findings were in support of our study.

In the present study 85.1% of the samples showed Gram negative organisms and 14.9% showed Gram positive organisms which gives the ratio of 5:1. This is supported by the study of **Oni A A et al** [62] in which they showed the ratio of Gram negative to Gram positive to be 4.6:1. Also it was shown in the present study that 78.6% were Gram negative aerobes and 21.4% were Gram negative anaerobes and among the Gram positive all the isolates were aerobes and there were no anaerobes. Similar finding by **Thanni et al** [84] also showed that 70.5% of the organisms were Gram negative aerobes and 27.5% were Gram positive

aerobes. **Appelbaum et al** [3] also demonstrated 15.8% Gram negative anaerobes in their study correlating with our study. Whereas **Gerding D N et al** [34] demonstrated most of the anaerobic isolates to be Gram positive, but all his specimens were from foot infections in diabetic patients. But our study involved not only the diabetic foot ulcers but also from ulcers of varied etiology and all our diabetic patients were of younger age group.

In the present study it was found that polymicrobial infection was common accounting for 81.7% aerobes and 18.3% anaerobes. Similarly **Brook I et al** [10] demonstrated polymicrobial wound infection in 76% of intra abdominal and post surgical abdominal wound infections, 32% in cutaneous abscesses and 69% in diabetic foot lesions. **Goldstein E J et al** [35] demonstrated 90.4% polymicrobial infections in human and animal bite wounds. **Panichi G D et al** [63] demonstrated 63.5% polymicrobial infection in abdominal infections. Thus polymicrobial infection is very common in all infected wounds which was also proved in the present study. The polymicrobial infection might be due to the ability of anaerobes to impaired host defense thereby allowing their copathogens to exert their intrinsic virulence, the provision of nutrients by one bacterial species to enhance the growth of bacterial partners, capacity of anaerobes to alter the local micro environment thereby rendering it more conducive

to bacterial survival and proliferation, transfer of virulence factors to other microbes causing mixed infections [15].

In the present study it was shown that Bacteroides fragilis was the common Gram negative organism isolated from wound infections (18.2%) especially from the Surgery ward (10.2%). Bacteroides fragilis was also isolated from the Burns ward (2.8%), Orthopaedics, Obstetrics & Gynaecology and Surgical Gastroenterology (1.7%) each. Thus Bacteroides fragilis is the common Gram negative anaerobe which was isolated in the wound infections. **Appelbaum et al** [3] also demonstrated Bacteroides fragilis as the common Gram negative anaerobe in 15.8% of their specimens. **Mittermayer H et al** [56] also demonstrated Bacteroides fragilis in 46.1% of infections originating from lower intestinal tract and bile duct. And **Beena V K et al** [6] demonstrated Bacteroides fragilis in 21.12% of human infections. **Neerja Jindal et al** [59] also demonstrated Bacteroides fragilis in 30.2% of non tuberculous empyema. **Saini et al** [70] demonstrated Bacteroides fragilis in 13.3% of pelvic inflammatory disease. All these are supporting our study. Most of the Bacteroides fragilis isolated in our studies were from post operative wound infections (8%), diabetic foot ulcers (6.2%) and in burns (1.1%), open injury following accidents (1.1%) and in decubitus ulcers, septicaemia and intra abdominal abscesses (0.5% each). It has been

clearly documented that Bacteroides fragilis group present as normal flora and play a role in initiating pathogenesis and act as a pathogen in diverse human infection. The high isolation rate of Bacteroides fragilis group in diabetic foot ulcers may be due to the predisposing factors like prolonged treatment, vascular insufficiency and the synergistic action of other bacteria which would have facilitated the multiplication of Bacteroides fragilis group [6]. The only Bacteroides fragilis isolate in septicaemia case in the present study was from the pelvic abscess following curettage of uterus, hence the probable site of entry of the organisms into the blood was through the uterine vessels. **Saini et al** [70] also had shown Bacteroides fragilis in 13.3% of pelvic inflammatory disease. **Bowler P G et al** [9] explained in their article on wound microbiology that the exposed burn wound is susceptible to microbes from the gastro intestinal and upper respiratory tract, especially the commonest commensal of gastro intestinal tract, Bacteroides fragilis. Similarly **Mousa et al** [57] also reported the presence of Bacteroides species in the wounds of 82% of patients who developed septic shock and concluded that such microbes might have played a significant role in burn wound sepsis. The Bacteroides fragilis isolated from the decubitus ulcer in the sacral region of the patient might have let into the skin erosion,

local tissue ischaemia and necrosis and it might have become susceptible to faecal contamination.

In the present study, it has been observed that, Bacteroides fragilis were isolated more commonly in the males (10.8%) in the age group 46 – 55 years and less commonly in females (7.4%) in the age group 26 – 35 years. **Bergan T et al** [7], **Chakravorti and Chatterjee et al** [14], have demonstrated that Bacteroides fragilis was very common in the females than the males which is contrary to our study. But they have demonstrated the isolation of Bacteroides fragilis in younger age group in females during active reproductive life of 26 – 35 years which is in support of our study. The variation in the sex distribution might be due to the selection of cases. The study by **Bergan T et al** [7] was based on female genital tract infections whereas in the present study not only included female genital tract infection but also other infections like post operative, diabetic foot ulcer, decubitus ulcer etc., in which the predisposing factors for wound infection is more common in males than females. The cause for the younger age group in females might be due to the infections emanating from the normal vaginal flora and outer cervical canal and contributed to colpitis and abscesses developing in the pelvic organs during the intervention processes [7].

In the present study it was shown that out of 133 pus samples, Bacteroides fragilis was isolated from 28 (21%) and Bacteroides fragilis was isolated from 2 (10%) out of 20 blood samples and from 2 (9%) out of 22 aspirate samples. Thus Bacteroides fragilis was commonly isolated from pus samples especially from General Surgical wards followed by blood samples and the aspirates. **Bowler PG et al [9]** in his article explained a wide variety of virulent factors in Bacteroides fragilis which were responsible for wound infections. He had explained that adhesion factors like capsular polysaccharides, exoenzymes like proteases, antiphagocytic factors like capsule, short chain fatty acids and immunoglobulin may contribute to the impairment of wound healing process and promoting a prolonged anaerobic environment and survival of Bacteroides fragilis in wounds. Hence identification of Bacteroides fragilis in pus samples is justified from wound. **Brook I et al [10]** also demonstrated that materials appropriate for anaerobic organisms like Bacteroides fragilis isolation were blood specimen and aspirates. In the present study also, Bacteroides fragilis was isolated from blood and aspirates.

The antimicrobial susceptibility testing of Bacteroides fragilis in the present study have shown that Bacteroides fragilis was sensitive to the drugs like carbenicillin (87.5%), cefaperazone (90.6%), chloramphenicol (96.8%), clindamycin (87.5%), metronidazole (96.8%), penicillin

(81.2%), cefotaxime (93.8%), tetracycline (90.7%). The resistance was 12.5% to carbenicillin and clindamycin, 9.3% to cefaperazone and tetracycline, 3.1% to chloramphenicol and metronidazole, 18.8% to penicillin, 6.2% to cefotaxime. **Chaudary R et al** [15] in their review article showed similar type of resistance for Bacteroides fragilis to these antimicrobials but he had shown all Bacteroides species were susceptible to metronidazole whereas we had one strain of Bacteroides fragilis which was resistant to metronidazole.

Edlund C et al [24] also demonstrated that Bacteroides fragilis was not resistant to metronidazole and chloramphenicol. **Dave J et al** [18] demonstrated a case of infection involving a prosthetic joint with the isolation of Bacteroides fragilis which was resistant to metronidazole. Similarly, **Haggoud A et al** [37], **Stubbs et al** [80], **Trinhs and Reysset et al** [85], **Gal M et al** [33], **Soki J et al** [78], **Jamal W Y et al** [45] had shown metronidazole resistant Bacteroides fragilis which are in support of our study. But the only isolate in the present study was from intra abdominal surgery. The resistance of Bacteroides fragilis to metronidazole may be due to the reduced uptake of the drug, reduced nitroreductase activitiy, decreased pyruvate: ferredoxin oxido reductase activity, increased lactate dehydrogenase activity or due to the transfer of resistance by 7.7 Kb plasmid and in few strains chromosomal.

The sequence analysis by short sequencing method done at MICROSYNTH LABORATORY, SWITZERLAND, revealed that the 16Sr DNA sequence was approximately 99% homology to Bacteroides fragilis genome. This establishes beyond doubt that biochemically proved strain is indeed Bacteroides fragilis. Similar study by **Jeffrey M Schapiro et al** [46] also showed the confirmation of the identity of Bacteroides fragilis by sequencing of 16S rDNA. The study by **Yamashita et al** [91] also revealed the identification of chromosomal DNA of Bacteroides fragilis by 16S rDNA sequence. Hence the 16S rDNA homology analysis is considered to be one of the confirmatory methodology for the biochemically proven Bacteroides fragilis identification.

The homology analysis between metronidazole sensitive Bacteroides fragilis and metronidazole resistant Bacteroides fragilis showed a difference of 2.4% denoting that they are separate strains. Similar study by **Yuli Song et al** [92] in his study on the evaluation of 16Sr DNA in the clinical identification of Bacteroides species revealed that if an isolate showed genetic difference of >1% and <2% that was closely related to its best match. Thus he proved that 21 of his isolates had 98.6% sequencing similarity. In the present study the genetic difference was more than 2%, hence it is confirmed that both the strains are different. As the two strains subjected for this analysis were the different

strains proved by the sensitivity pattern as sensitive and resistant. It can be confirmed that the only isolate from the Surgical ward which was metronidazole resistant is proved to be the resistant strain beyond doubt.

SUMMARY

Bacteroides fragilis has emerged out as an important anaerobic organism contributing to infections in Surgical wards. Among them, resistance to metronidazole was also observed. Though metronidazole resistant organisms were isolated in a few Indian series, no such work has been carried out on the genomic pattern of resistant organisms.

The present study on **Bacteroides Fragilis in Sepsis – Microbiological studies** was attempted to find out the prevalence of Bacteroides fragilis in Surgical samples and to perform antimicrobial profile of these organisms in order to segregate metronidazole resistant samples from others and process the same at an International Centre for gene sequencing. Finally it was proposed to give guidelines to clinicians dealing with such cases of pyogenic infections.

With the rigid criteria for inclusion and exclusion, this study was carried out at Government Rajaji Hospital, Madurai, among the patients admitted to Surgical wards after clearance from Institutional Ethical Board and after informed consent. Advanced microbiological studies with reference to Bacteroides fragilis was carried out at the Department of Immunology, The American College, Madurai and Cancer Biology, School of Biological Sciences, Madurai Kamaraj University. The internal quality control was maintained at all levels.

Bacteroides fragilis was isolated from 32 out of 175 samples and the prevalence was 18.3%. The isolates were more from post operative wound infections which constituted 8%. The isolates were more from males (M:F = 2:1) and it was attributed to their behaviour.

Many of the isolates were resistant to multi drugs, but only one of them was resistant to metronidazole. This resistant isolate along with a metronidazole sensitive isolate were subjected to genomic sequencing. The homology of the resistant strain was different by 2.4% with the sensitive strain, thus confirming the resistance pattern genomically.

In view of the emerging multidrug resistance pattern among Bacteroides fragilis, the clinicians were informed about the pattern of resistance and the need for polyantimicrobial therapy in deserving cases on clinical and laboratory grounds.

CONCLUSION

This study on **Bacteroides Fragilis in Sepsis – Microbiological studies** among patients with sepsis from General Surgical, Surgical Gastroenterology, Burns, Orthopaedics and Obstetrics & Gynaecology wards revealed

- Bacteroides fragilis was isolated from 32 of 175 samples processed. Thus the prevalence was 18.3%
- The prevalence was high among the post operative wound infections (8%) commonly in the males in the age group 46 – 55 years and in the females in the age group 26 – 35 years, from the pus samples.
- Of the 32 samples, antimicrobial susceptibility testing of Bacteroides fragilis revealed only one of the organism was resistant to metronidazole.
- Genomic sequence of metronidazole resistance in an International Laboratory revealed that the homology of the resistant strain showed a difference of 2.4% with the sensitive strain, thus confirming the resistant pattern.
- The patient with metronidazole resistant Bacteroides fragilis infection has improved well with the combination of clindamycin with other antimicrobials.

- The resistant organism was isolated from a post operative wound infection thus giving a warning signal to the clinicians on emerging metronidazole resistance on nosocomial anaerobic infections in this hospital.
- In view of the emerging infections, surgeons were informed about the inclusion of clindamycin in the management of post operative wound infection.

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APPENDIX I – PATIENT INFORMATION PROFORMA

Name : Address:

Age :

Sex:

Occupation:

Education:

Income:

IP No:

Ward No:

Diagnosis:

Date of admission:

Date of surgery:

Date of discharge:

Complaints of:

Fever: Continuous, Intermittent, Low grade, High grade,
Associated with Chills / Sweating

Cough: Productive /non Productive,

Discharge : Colour, Quantity, Nature - watery, purulent, blood stained, foul smelling,

Pain: Nature, Intensity, Duration

Present History:

Past History: H/O DM, HT, Anaemia, Jaundice, Convulsions

General Examinations: H/O of ear discharge, conjunctivitis, trauma, anaemia, clubbing, cyanosis, icterus, generalized lymphadenopathy, skin, hair, nail changes

Systemic examination:

CVS:

RS:

Per abdomen:

CNS:

Treatment given previously:

Treatment given as per antibiogram:

Outcome:

Cured / Severity reduced / Worsened / Death

Lab investigations:

Specimen:

Lab number:

Date:

Time:

APPENDIX II – LAB INVESTIGATION PROFORMA

Name:

Age:

Sex:

Provisional Diagnosis:

Specimen: 1. Nature

2. Source

Date of Collection:

Date of Transport:

Date of Reception:

Mode of Transport of Specimen:

Macroscopic Examination:

Microscopic Examination:

Culture:

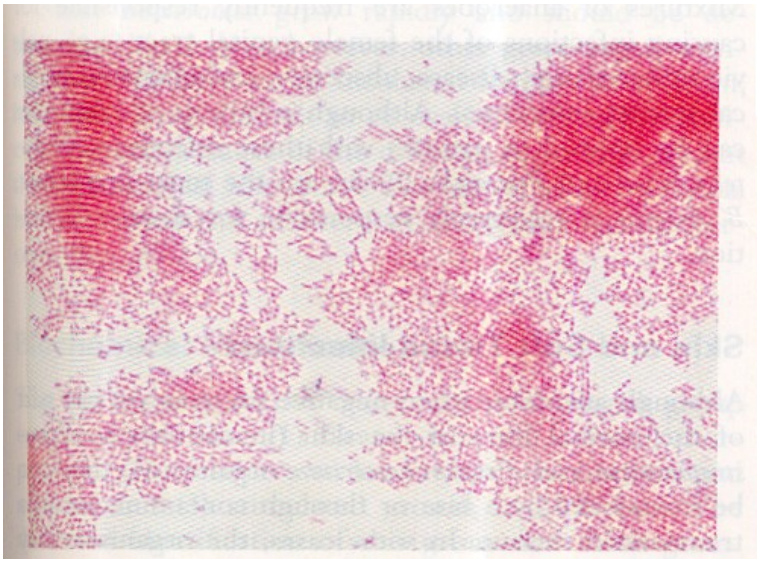
Biochemical Reactions:

Antibiotic Susceptibility Testing:

Sequence Analysis:

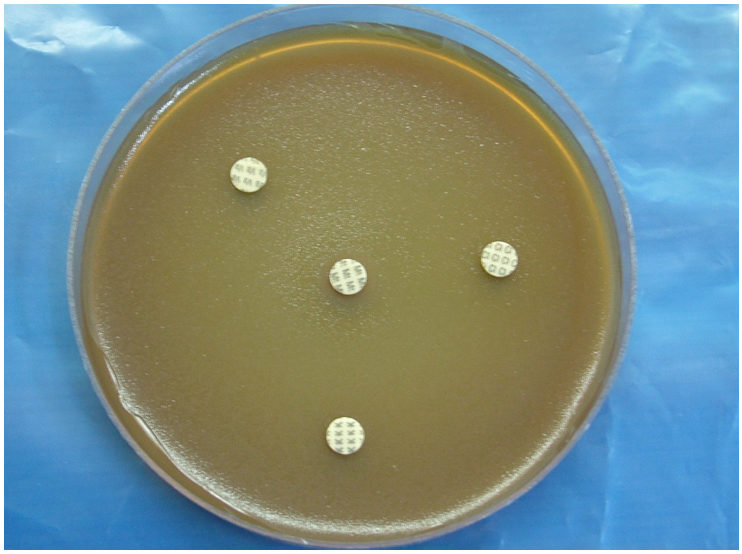
SPECIMEN PROCESSING

GRAM STAINING



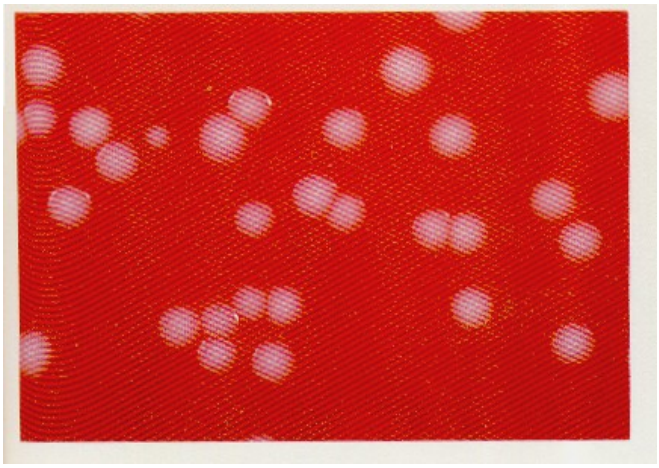
Pleomorphic pale staining Gram negative rods

SPECIAL POTENCY ANTIOBIOTIC DISC



Resistant to Kanamycin, Colistin, Vancomycin

5% SHEEP BLOOD AGAR PLATE



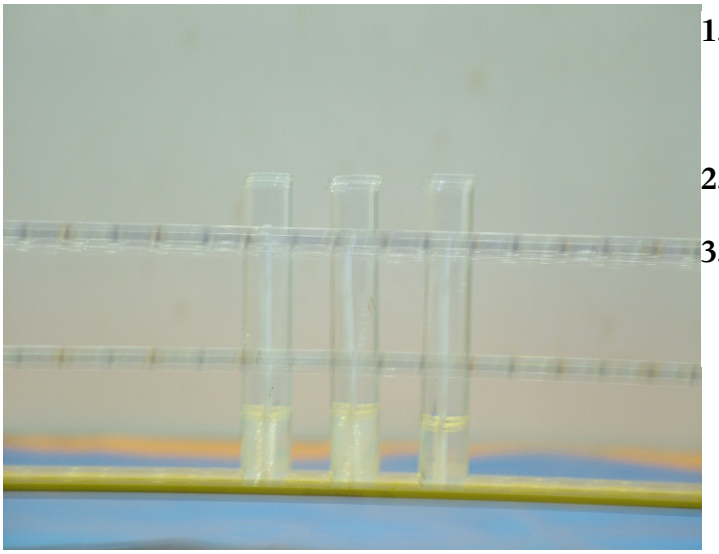
Grey white, glistening, nonhaemolytic colonies

GASPAK ANAEROBIC JAR WITH ANAEROGAS PACK

BILE ESCULIN AGAR WITH KANAMYCIN

Grey Circular raised colonies with stippling in the medium around the colonies (bile resistant and esculin hydrolysis)

CATALASE TEST



2 1 3

1. **TEST – POSITIVE**

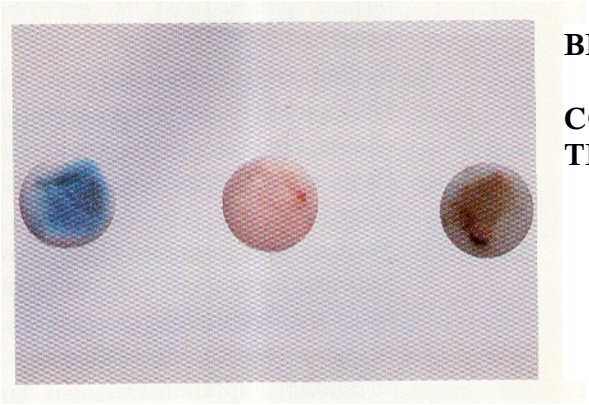
   (brisk effervescence)

2. **POSITIVE CONTROL**

3. **NEGATIVE CONTROL**

   (no effervescence)

SPOT INDOLE TEST



BLUE – POSITIVE

COLOURLESS OR OTHER THAN BLUE - NEGATIVE

16 S rDNA AMPLIFIED BY PCR